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Effect of Amino Acid Availability on Vitamin B_{12} Production in *Lactobacillus reuteri*▽†

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Recent functional genomics and genome-scale modeling approaches indicated that B_{12} production in *Lactobacillus reuteri* could be improved by optimization of the medium. Here we show that a series of systematic single-amino-acid omissions could significantly modulate the production of B_{12} from nearly undetectable levels (with omission of isoleucine) to levels 20-fold higher than the levels previously reported (with omission of cysteine). Using cDNA microarray experiments, we analyzed the transcriptional response of *L. reuteri* to medium lacking cysteine. The results supported the observed high level of B_{12} production and provided new avenues for future improvement of production of vitamin B_{12}.

Vitamin B_{12} analogues (generally termed B_{12}) form a diverse subgroup of the tetrapyrroles and are structurally complex molecules that contain a ring-contracted porphinoid with cobalt chelated at the core (5, 10). Synthesis of B_{12} is restricted to a few clades of bacteria and archaea, while vitamin B_{12} auxotrophies are widespread in prokaryotes, protists, and animals, including humans (11).

Vitamin B_{12} is a relevant compound from an anthropocentric perspective. It is an essential dietary compound (vitamin) with a recommended intake of 2.4 μg/day for healthy adults (4), and vitamin B_{12} deficiency has been associated with several pathologies, including different forms of anemia and neurological dysfunction, among others (19). Additionally, it is also an industrially relevant compound since it is an essential cofactor in reactions used in biotechnological processes, such as the production of 1,3-propanediol (2).

Lactobacillus reuteri is a heterofermentative lactic acid bacterium that colonizes the gastrointestinal tracts of humans and other animals (23). It has been shown to contain a functionally active B_{12} biosynthetic gene cluster that encodes all the enzymes required for the synthesis of this important cofactor from 5-aminolevulinate (12, 14). In a B_{12}-dependent reaction catalyzed by glycerol dehydratase (EC 4.2.1.30), *L. reuteri* is able to synthesize 3-hydroxypropanaldehyde (reuterin) (20). This compound, which has broad-spectrum antimicrobial activity (1), can be further reduced to 1,3-propanediol, restoring NAD^+, if the reaction is coupled to the oxidation of another carbon source.

We have recently studied glucose and glycerol cofermentation by *L. reuteri* using functional genomics and genome-scale modeling techniques. This enabled us to broaden our view of the physiological responses of *L. reuteri* to glycerol, which has strong implications for amino acid metabolism and B_{12} biosynthesis (12). Here, we focused on these new targets and attempted to engineer the production of B_{12} in *L. reuteri* using physiological approaches. This led to the discovery that omission of single amino acids can significantly affect the production of B_{12} in *L. reuteri*. Hence, we studied the genome-wide transcriptional response of *L. reuteri* to the omission of cysteine in a search for mechanistic insights and new leads for metabolic engineering.

MATERIALS AND METHODS

Strain, media, and culture conditions. We obtained *L. reuteri* JCM1112 (type strain, human isolate) from the Japanese Collection of Microorganisms (Riken, Japan). It was cultured at 37°C in a version of chemically defined medium (CDM) from which vitamin B_{12} was omitted (22). When appropriate, 0.5% glycerol was added. Cells were cultivated in standard nonstirred batch cultures under an air atmosphere, unless stated otherwise. When mentioned below, oxygen availability was restricted by cultivating *L. reuteri* in anaerobic jars filled with either O_2-free N_2 or a mixture of 95% N_2 and 5% CO_2.

Single-omission growth experiments. We investigated the effect of omitting each amino acid in CDM separately on the B_{12} production by and growth behavior of *L. reuteri*. Parallel experiments were performed in the presence and absence of glycerol, omitting an amino acid in each culture and using a culture with no omission as a reference. Independent duplicate experiments were performed as described below. An overnight culture of *L. reuteri* washed twice in 0.85% (wt/vol) NaCl was used to inoculate all media to obtain an initial optical density at 600 nm (OD_{600}) of 0.05. Batch cultures were propagated using five consecutive transfers in the corresponding media, monitoring the growth every 24 h. After 48 h, if there had not been at least two doublings (OD_{600}, <0.2), the culture was discarded and not used for further analysis. If after the fourth transfer the OD_{600} was >0.5 after 24 h of incubation, the culture was used to inoculate 20 ml of the corresponding medium. The resulting culture, the last culture, was used to determine the maximum specific growth rate (μ_{max}) and the B_{12} content.

Growth rate determination. *L. reuteri* JCM1112 was cultivated in several variations of CDM in 96-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands). Different replicates were placed on the plate using a checkerboard format for distribution (3). The plates were incubated at 37°C in a Genios microtiter plate reader (Tecan, Zurich, Switzerland) set to monitor growth by measuring the OD_{595} every 15 min. All measurements were obtained independently at least twice for at least eight biological replicates. μ_{max} was determined

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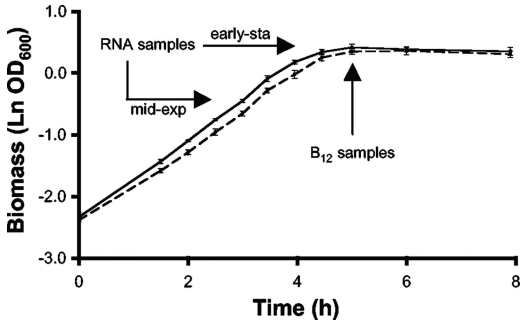

FIG. 1. Biomass formation and sampling scheme for pH-controlled batch fermentations of CDM in the presence (solid line) or absence (dashed line) of cysteine. mid-exp, mid-exponential growth phase; early-sta, early stationary growth phase.

in a high-throughput fashion by in-house scripts that calculated the growth rate for every five consecutive time points throughout the growth curve and showed the μ_{max} per well.

Coenzyme B_{12} determination. B_{12} production by *L. reuteri* in the different CDM variations was determined as described in the *Official Methods of Analysis of AOAC International*, using the *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830 vitamin B_{12} assay (6). Cell extracts used for B_{12} analysis were prepared as described elsewhere (13).

Fermentation conditions. pH-controlled batch fermentations of *L. reuteri* in the presence or absence of cysteine were carried out using an experimental setup consisting of four vessels with a reaction volume of 400 ml. Prior to inoculation, the vessels containing medium were gassed with O_2-free N_2 (15 ml/min) for 1 h. The temperature was kept constant at 37°C, and the pH was kept at 5.8 by titration with 5 M NaOH. Homogeneity was ensured by continuous stirring throughout fermentation. An exponentially growing culture in the corresponding medium was used to inoculate the fermentors to obtain an initial OD_{600} of 0.05. Periodic measurements of OD_{600} were used to monitor biomass formation. Samples used for transcriptome analysis were harvested at mid-logarithmic growth phase (OD_{600}, 1) and in early stationary phase, 15 min after exponential growth ceased (Fig. 1). At this stage of the growth curve, we also obtained samples for B_{12} analysis (Fig. 1).

Microarray design. Dedicated microarrays for *L. reuteri* JCM1112 were spotted using the high-density Agilent 44K platform (Agilent Technologies, Santa Clara, CA), based on the draft genome sequence of *L. reuteri* JCM1112 released by JGI (retrieved in March 2006). We used a custom probe design covering 1,700 of 1,900 predicted coding regions (~90% coverage) that we had developed for the 11K format available at GEO (accession number GPL6856; http://www.ncbi.nlm.nih.gov/geo) and had it printed fourfold per array. Oligonucleotides were designed to probe the predicted coding regions; 82.9% of the coding regions were represented by 24 or more probes (6 unique probes), and only 3.9% of the coding regions were represented by 12 or fewer probes (3 unique probes).

RNA isolation. Cells harvested from cultures grown in the presence or absence of cysteine were sampled by rapid quenching using a cold methanol method (9). Extraction and purification of total RNA were carried out as previously described (12). The RNA concentration was determined with an ND-1000 spectrophotometer (NanoDrop Technologies Inc., United States). The integrity of mRNA species was confirmed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). A threshold for the 23S/16S RNA ratio of 1.6 was used for samples to be considered satisfactory. Only samples with ratios greater than this value were used for transcriptome analysis.

cDNA synthesis and labeling. For each sample, 20 μg of RNA was used for first-strand cDNA synthesis using Superscript III reverse transcriptase (Invitrogen, Breda, The Netherlands) according to the recommendations of the manufacturer. All samples of newly synthesized cDNA were purified and labeled with cyanine 3 and cyanine 5. Indirect labeling was performed with a CyScribe first-strand cDNA labeling kit (Amersham, United Kingdom) by following the manufacturer's recommendations. cDNA concentrations and label incorporation were verified using the ND-1000 spectrophotometer (NanoDrop Technologies Inc., United States).

Microarray hybridization. We used 0.8 μg of cDNA labeled with each dye for each hybridization. All samples were hybridized with each label at least once to facilitate recognition of possible dye effects. A hybridization scheme (see the supplemental material) consisting of a loop design comprising 13 microarrays was used to scrutinize the transcriptomes of mid-logarithmic- and early-stationary-growth-phase cells cultured in pH-controlled batch fermentations in CDM containing or not containing cysteine. The hybridization mixtures were incubated at 60°C for 17 h, after which the slides were washed according to the recommendations of the manufacturer. Scanning took place immediately after the slides were dried as described elsewhere (16).

Scanning and microarray data analysis. Slides were scanned with a ScanArray Express scanner (Perkin-Elmer) set to maximum resolution (5 μm). ImaGene (version 5.6; BioDiscovery) was used for image analysis, spot quantification, and data extraction. After normalizing the transcriptome data by local fitting an M-A plot applying the loess algorithm (24) using the Limma package (17) in R (http://www.r-project.org), we analyzed the transcriptome data as described elsewhere (16). Statistical significance was tested using the difference between biological duplicates by implementation of the eBayes function included in Limma (cross-probe variance estimation) and false discovery rate adjustment of the *P* values (18). Two comparisons were used to characterize the impact on *L. reuteri* of removing cysteine (i) during exponential growth phase (mid-logarithmic-phase response) and (ii) during early stationary growth phase (early-stationary-phase response). Transcripts were considered for analysis if the *P* value was less than 0.05 and the absolute $\log_2$ ratio was greater than 0.585. The transcriptome data were visualized by projection on metabolic maps of the genome-scale model developed previously for *L. reuteri* (12).

Microarray accession numbers. The microarray platform developed in this study is available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession number GPL7505. The microarray data obtained have been deposited in the same database under accession number GSE13391.

RESULTS

We recently observed that cultivation of *L. reuteri* in a CDM with glycerol has profound implications for B_{12} production and amino acid metabolism (12). Here, we followed these new leads and attempted to modulate the production of B_{12} by changing the amino acid composition of CDM and describing the phenotypic changes related to growth and to the production of this cofactor observed.

Single-amino-acid omissions and growth of *L. reuteri*. We found that eight amino acids could be omitted from CDM without completely inhibiting the growth of *L. reuteri*. These omissions affected the growth rate and the final biomass formed to different degrees (Table 1). The omission of serine (Ser) profoundly reduced the specific growth rate and final optical density both in the absence and in the presence of glycerol. In CDM lacking isoleucine (Ile) the final amount of biomass formed was affected much more than the growth rate. Omission of the other amino acids, namely alanine (Ala), aspartate (Asp), cysteine (Cys), glycine (Gly), lysine (Lys), and proline (Pro), only slightly affected final amount of biomass formed. The growth rates measured for all single-amino-acid omissions increased when glycerol was added, except for CDM lacking Gly, in which the growth rate was only slightly affected (6% reduction).

Single-amino-acid omissions and vitamin B_{12} production. *L. reuteri* JCM1112 produces approximately 20 μg liter^{-1} $OD_{600}{}^{-1}$ of B_{12} in complete CDM without glycerol. If glycerol is added, B_{12} production by *L. reuteri* increases about fivefold to 100 μg liter^{-1} $OD_{600}{}^{-1}$. When B_{12} production in *L. reuteri* cultures grown in complete CDM was compared with B_{12} production in *L. reuteri* cultures grown in CDM lacking single amino acids, very different effects were observed (Table 2). The results for omission of Cys are the most remarkable since, even without addition of glycerol, omission of Cys leads to a fivefold increase in B_{12} production compared to that in complete CDM. Furthermore, B_{12} synthesis can be further in-

TABLE 1. Growth of *L. reuteri* in single-amino-acid-deficient CDM supplemented or not supplemented with glycerol

Amino acid omitted	Concn (mM)[a]	Growth in CDM[b]		Growth in CDM with glycerol[b]		Difference between μ_{max} in CDM and μ_{max} in CDM with glycerol (%)
		μ_{max} (h^{-1}) (%)	Final OD_{595} (%)	μ_{max} (h^{-1}) (%)	Final OD_{595} (%)	
None	NA	0.453 (100)	1.21 (100)	0.575 (100)	1.29 (100)	27
Ala	2.69	0.368 (81)	1.17 (97)	0.519 (90)	1.31 (102)	41
Asp	3.16	0.445 (98)	1.20 (99)	0.551 (96)	1.31 (102)	24
Cys	0.83	0.387 (85)	1.16 (96)	0.512 (89)	1.26 (98)	32
Gly	2.33	0.370 (82)	1.17 (97)	0.349 (61)	1.21 (94)	−6
Ile	1.60	0.356 (79)	0.41 (34)	0.411 (72)	0.46 (36)	15
Lys	2.68	0.433 (96)	1.20 (99)	0.540 (94)	1.27 (98)	25
Pro	5.86	0.424 (94)	1.19 (98)	0.555 (97)	1.28 (99)	31
Ser	3.24	0.106 (23)	0.25 (21)	0.131 (23)	0.24 (19)	24

[a] Concentration of amino acid in CDM when it was present. NA, not applicable.
[b] Each value is the average (percentage of the value for the same conditions when no amino acid was omitted) for at least eight biological replicates. Each experiment was repeated twice, and similar results were obtained.

creased by the presence of glycerol, which has a stimulatory effect, resulting in a level of more than 350 μg liter^{-1} $OD_{600}{}^{-1}$.

The increase in B_{12} production due to addition of glycerol was observed regardless of the amino acid omitted from CDM, although the proportions varied. The greatest stimulatory effect of glycerol (20-fold) occurs when Ile is omitted. However, this is due to the sharp reduction (>10-fold) in B_{12} production to levels that are barely detectable when glycerol is absent rather than to an increase in B_{12} production in CDM with glycerol.

Besides the effects mentioned above, in the absence of glycerol *L. reuteri* produced larger amounts of B_{12} in CDM lacking Lys, Ala, Gly, and Asp, while in the presence of glycerol only the omission of Ala and Asp had a positive effect.

Effect of oxygen on B_{12} production. Cysteine is known to be a potent reducing agent. Omission of cysteine leads to an increased redox potential and oxygen availability in the growth medium. The concentration of oxygen has been reported to affect the production of B_{12} in propionibacteria negatively (7).

TABLE 2. Vitamin B_{12} production by *L. reuteri* in single-amino-acid-deficient CDM supplemented or not supplemented with glycerol

Amino acid omitted	Amt of B_{12} (μg liter^{-1} $OD_{600}{}^{-1}$) in[a]:		Change (fold) from CDM to CDM with glycerol	Change (fold) compared to reference conditions[b]
	CDM	CDM with glycerol		
None	21.2 ± 3.6 (100)	105.0 ± 17.0 (100)	5	5
Ala	43.2 ± 4.7 (204)	143.3 ± 10.9 (136)	3	7
Asp	29.0 ± 4.8 (137)	146.8 ± 10.4 (140)	5	7
Cys	103.2 ± 13.0 (488)	351.8 ± 28.4 (335)	3	17
Gly	33.0 ± 3.3 (156)	113.9 ± 14.1 (108)	3	5
Ile	2.1 ± 0.4 (10)	42.4 ± 5.8 (40)	20	2
Lys	50.5 ± 5.1 (239)	114.0 ± 22.5 (109)	2	5
Pro	22.0 ± 4.7 (104)	72.6 ± 11.9 (69)	3	3
Ser	16.1 ± 3.6 (76)	76.0 ± 10.4 (72)	5	4

[a] The values are averages ± standard deviations (percentages of the value for the same conditions with no amino acid omitted) for at least three technical replicates. Each experiment was repeated twice, and similar results were obtained.
[b] The reference conditions are CDM without glycerol added and with no amino acid omitted.

For this reason, we decided to check whether varying the oxygen availability could alter the stimulatory effect of omission of Cys on B_{12} production in *L. reuteri*. This was done by cultivating *L. reuteri* in parallel under an air atmosphere and in an anaerobic vessel containing either pure N_2 or a mixture of 95% N_2 and 5% CO_2 and checking B_{12} production with all possible variations of CDM combining the presence and absence of glycerol and cysteine (Fig. 2). In the presence of 100% N_2, the production of B_{12} was slightly increased, by about 30%. The only exception was when cysteine was absent and glycerol was present, conditions under which there was a trend but no significant increase ($P > 0.05$). Cultivation in the presence of 95% N_2 with 5% CO_2 caused the B_{12} content to increase slightly more, by about 50%. The trend observed for the cultures in which the absence of cysteine was combined with the presence of glycerol is even more pronounced. Under these conditions B_{12} production increased to 428 ± 40 μg liter^{-1} $OD_{600}{}^{-1}$.

Transcriptomics of *L. reuteri* in the absence of cysteine. To obtain further insight into the stimulatory effect of Cys on B_{12} production, we studied the genome-wide transcriptional response of *L. reuteri* to cultivation in the absence of Cys by developing and performing cDNA microarray experiments. We determined the specific responses in mid-exponential and

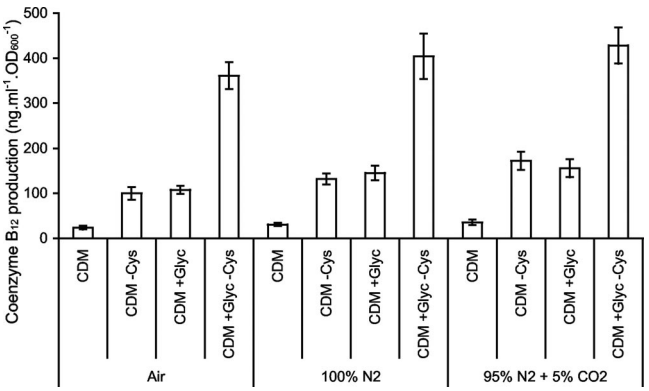

FIG. 2. Vitamin B_{12} production by *L. reuteri* with different gas environments in variants of CDM. −Cys, cysteine omitted; +Glyc, supplemented with glycerol.

TABLE 3. Some differentially regulated genes[a]

Locus	Product	Log_2 value[b]	
		M_{exp}	M_{sta}
Amino acid transport and metabolism			
lreu_0190	Succinyl-diaminopimelate desuccinylase (EC 3.5.1.18)		−0.70
lreu_0293	Cystathionine beta-lyase (EC 4.4.1.8)/cystathionine gamma-lyase (EC 4.4.1.1)	1.72	
lreu_0294	Cystine transport system permease protein	1.94	3.23
lreu_0295	Cystine transport ATP-binding protein	2.26	3.16
lreu_0348	Aspartate aminotransferase (EC 2.6.1.1)		−0.87
lreu_0377	Succinyl-diaminopimelate desuccinylase (EC 3.5.1.18)		−1.24
lreu_0425	Ornithine carbamoyltransferase (EC 2.1.3.3)	−3.38	−0.69
lreu_0426	Carbamate kinase (EC 2.7.2.2)	−2.27	
lreu_0445	Arginine deiminase (EC 3.5.3.6)	−2.09	
lreu_0502	Cysteine desulfurase (EC 2.8.1.7)/selenocysteine lyase (EC 4.4.1.16)		1.16
lreu_0610	Diaminopimelate epimerase (EC 5.1.1.7)		−1.07
lreu_0611	Aspartokinase (EC 2.7.2.4)		−1.51
lreu_0612	Diaminopimelate decarboxylase (EC 4.1.1.20)		−0.70
lreu_0613	Tetrahydrodipicolinate *N*-acetyltransferase (EC 2.3.1.89)		−0.65
lreu_1553	Cysteine synthase (EC 2.5.1.47)	1.27	2.06
lreu_1791	Homoserine *O*-succinyltransferase (EC 2.3.1.46)	0.80	1.27
lreu_1792	Cysteine synthase (EC 2.5.1.47)	0.96	1.16
Nucleotide transport and metabolism			
lreu_0123	Aspartate carbamoyltransferase (EC 2.1.3.2)		−5.32
lreu_0124	Dihydroorotase (EC 3.5.2.3)		−5.36
lreu_0125	Dihydroorotate dehydrogenase, catalytic subunit (EC 1.3.3.1)		−5.09
lreu_0126	Orotidine 5′-phosphate decarboxylase (EC 4.1.1.23)		−4.91
lreu_0127	Orotate phosphoribosyltransferase (EC 2.4.2.10)		−4.82
Coenzyme transport and metabolism			
lreu_0510	Folylpolyglutamate synthase (EC 6.3.2.17)/dihydrofolate synthase (EC 6.3.2.12)		−2.33
lreu_0878	Diaminohydroxyphosphoribosylaminopyrimidine deaminase (EC 3.5.4.26)/5-amino-6-(5-phosphoribosylamino)uracil reductase		−0.92
lreu_0879	Riboflavin synthase alpha chain (EC 2.5.1.9)		−0.81
lreu_0880	GTP cyclohydrolase II (EC 3.5.4.25)/3,4-dihydroxy-2-butanone-4-phosphate synthase (EC 4.1.2.-)		−0.75
lreu_1279	2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3)		−0.63
lreu_1280	Dihydroneopterin aldolase (EC 4.1.2.25)		−0.73
lreu_1710	Precorrin-2 C_{20}-methyltransferase (EC 2.1.1.130)		0.71
lreu_1711	Sirohydrochlorin cobaltochelatase (EC 4.99.1.3)		0.72
lreu_1712	Uroporphyrin-III C-methyltransferase (EC 2.1.1.107)/uroporphyrinogen III synthase (EC 4.2.1.75)		0.60
Posttranslational modification, protein turnover, and chaperones			
lreu_0324	Glutaredoxin		0.71
lreu_0353	10-kDa chaperonin GroES		−0.83
lreu_0354	60-kDa chaperonin GroEL		−0.84
lreu_0376	Thioredoxin reductase (EC 1.8.1.9)	1.11	0.67
lreu_0539	Thioredoxin	1.45	0.96
Inorganic ion transport and metabolism			
lreu_1707	Cobalt transport protein CbiQ		0.85
lreu_1708	Cobalt transport protein CbiN		0.63
lreu_1709	CbiM protein		0.69

[a] See Table S1 in the supplemental material for a complete list.
[b] Log_2 (intensity of signal in the absence of cysteine/intensity of signal in the presence of cysteine) determined during mid-exponential growth phase (M_{exp}) and early stationary growth phase (M_{sta}).

early stationary growth phases. A complete list of loci that were found to be differentially regulated is shown in Table S1 in the supplemental material; the small number of loci that are discussed below are shown in Table 3.

We found 140 genes that were differentially regulated in mid-exponential phase; only 8 of these genes were found to be downregulated, while 132 of them were upregulated. For genes differently regulated in the stationary growth phase, we found 294 genes that were differentially regulated, 125 of which were downregualted and 169 of which were upregulated. A total of 58 genes occur in both data sets, while 82 genes were differentially regulated exclusively in exponential phase and 236 genes were specific for stationary phase.

In order to facilitate analysis of the transcriptome data, we

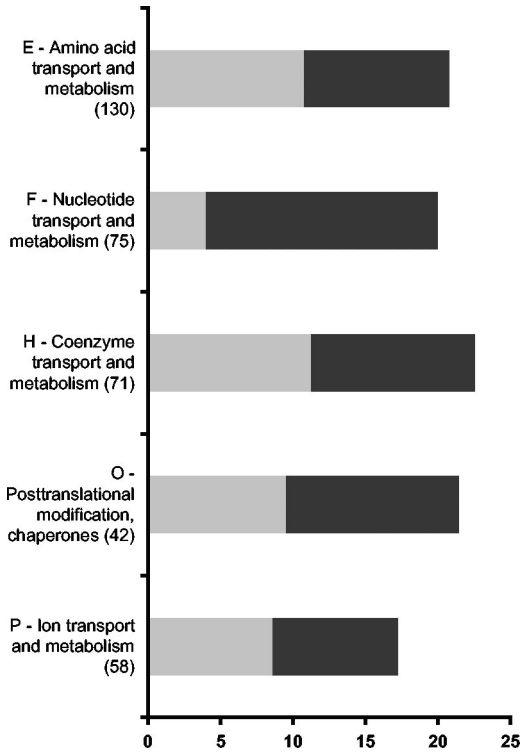

FIG. 3. Distribution of transcripts whose expression was affected by the absence of cysteine during early stationary growth phase for a selection of COGs (see Table S2 in the supplemental material for the complete distribution). Transcripts were considered to be differentially regulated for absolute M values of >0.585 and P values of <0.05. Light and dark gray bars indicate the proportions of upregulated transcripts in the absence and in the presence of cysteine, respectively. The total numbers of genes from the genome of *L. reuteri* included in the corresponding COGs are indicated in parentheses.

examined the distribution of the genes found to be differentially regulated according to the functional classes of the predicted encoded proteins, using the COG classification system (21). The complete distribution is shown in Table S2 in the supplemental material, and based on relevance for the topics discussed here some of the genes are described below (Fig. 3).

Not surprisingly, the COG category associated with amino acid transport and metabolism was overrepresented in both mid-exponential phase and stationary phase. Specific attention was given to genes involved in cysteine metabolism. There are two genes (lreu_1553 and lreu_1792) annotated in the chromosome of *L. reuteri* that are predicted to encode cysteine synthase (EC 2.5.1.47), and both of them are upregulated in the absence of cysteine. The same is true for the genes (lreu_0293 to lreu_0295, lreu_0502, and lreu_1791) coding for enzymes involved in the metabolism of sulfur-containing compounds, such as cysteine desulfurase (EC 2.8.1.7), selenocysteine lyase (EC 4.4.1.16), and cystathionine beta- and gamma-lyases (EC 4.4.1.8 and EC 4.4.1.1, respectively), among others. Genes predicted to encode enzymes related to arginine, aspartate, and ornithine metabolism tended to be downregulated (lreu_0425, lreu_0426, and lreu_0445). The latter genes are all involved in the synthesis of carbomyl phosphate, a precursor of pyrimidines.

In accordance, genes associated with the COG category that includes proteins assigned to nucleotide transport and metabolism not only are overrepresented but also display a clear tendency to be downregulated. Indeed, a closer look clearly shows that a complete cluster from lreu_0123 to lreu_0127 is drastically downregulated in the absence of cysteine (M value [$\log_2$ value of the signal intensity in the absence of cysteine divided by the signal intensity in the presence of cysteine], ~ -5).

Cofactors such as folate derivatives are important in the synthesis of DNA and RNA precursors. The folate biosynthesis gene cluster (lreu_0510 and lreu_1279-lreu_1280) and the gene cluster for biosynthesis of riboflavin (lreu_0878 to lreu_880) appear to be downregulated when cysteine is omitted from CDM and account for roughly one-half of the regulated genes assigned to coenzyme transport and metabolism (see Table S1 in the supplemental material). As expected, the B_{12} biosynthesis gene cluster was upregulated.

Overrepresentation was observed for genes encoding proteins assigned to the COG category related to posttranslational modification and chaperones. Again, the split between up- and downregulated genes appears to be quite even, implying that the absence of cysteine does not affect all chaperones indiscriminately. Among others, in the downregulated group there are loci predicted to encode the GroES-GroEL cochaperonin complex (lreu_353 and lreu_354), while in the upregulated group there are loci predicted to encode thioredoxin, thioredoxin reductase (EC 1.8.1.9), and glutaredoxin (lreu_539, lreu_0376, and lreu_0324, respectively).

One final category worth highlighting is the ion transport and metabolism proteins. In this category, most genes were downregulated, which can be ascribed mainly to the downregulation of nonspecific ABC transporters. The finding that the upregulated group includes genes involved in scavenging of cobalt from the environment (lreu_1707 to lreu_1709) is highly relevant.

DISCUSSION

The average B_{12} content of an early-stationary-phase culture of *L. reuteri* JCM1112 in CDM is approximately 20 μg liter $^{-1}$ $OD_{600}{}^{-1}$. The level increases about fivefold if CDM is supplemented with glycerol. Under these conditions, *L. reuteri* uses glycerol to recycle NAD^+ via conversion of glycerol to 3-hydroxypropanaldehyde, which is subsequently reduced to 1,3-propanediol (2). Cofermentation of glucose and glycerol by *L. reuteri* has recently been reported to have a major effect on amino acid metabolism (12). In this study, we determined the effect on growth behavior and B_{12} production of omitting individual amino acids present in CDM. This was determined in parallel in the presence and in the absence of glycerol.

In this screening exercise, we identified eight amino acids that are essential for *L. reuteri* to grow at least two generations in the first 2 days after inoculation. The well-established positive effect of glycerol on the growth rate of and B_{12} production by *L. reuteri* cultivated in complete CDM was observed regardless of the single amino acid omitted, with the exception of glycine (Table 1). When glycine is omitted, addition of glycerol to CDM still has a positive effect on B_{12} production (Table 2), but the effect on μ_{max} is lost. An explanation for this might be

the fact that the rate of consumption of glycine in the presence of glycerol is nearly twice that in its absence (12). This indicates that there is an increase demand for glycine in the presence of glycerol, which cannot be met when *L. reuteri* has to rely solely on de novo synthesis. This hypothesis is in agreement with the sharp decrease in the μ_{max} observed in the absence of glycine and glycerol for both *L. reuteri* and *Lactobacillus plantarum* (22).

In the absence of glycerol, the omission of several single amino acids leads to important increases in B_{12} production (for instance, a 40% increase for aspartate, a 60% increase for glycine, a 200% increase for alanine, and a 240% increase for lysine). Any of these findings by itself could lead to great improvements in B_{12} production processes, especially the omission of lysine since it has very little impact on the growth of *L. reuteri*. However, these findings are completely overshadowed by the 500% increase in B_{12} production caused by omission of cysteine alone. Combined with the stimulatory effect of glycerol, a 17-fold increase in B_{12} production was obtained (Table 2).

We have attempted to obtain mechanistic insights into and new clues for how to further increase the synthesis of B_{12} in *L. reuteri* by studying its genome-wide transcriptional response to the omission of cysteine. Due to the properties of cysteine as a reducing agent mentioned previously, we started by carrying out an exploratory experiment under controlled oxygen availability conditions. When cultured anaerobically, *L. reuteri* was the first organism reported to produce exclusively pseudovitamin B_{12}, providing a great advantage for downstream processing during its purification. Under strictly anaerobic conditions, B_{12} production by *L. reuteri* was increased by little more than 30%. This could be anticipated, since *L. reuteri* has been shown to encode the oxygen-independent biosynthetic route for B_{12} (14) and the initial step in industrial production of B_{12} using propionibacteria is carried out under strictly anaerobic conditions (7). However, the increase due to the absence of cysteine from the medium was not diminished under anaerobiosis, implying that this amino acid does indeed have other effects on the metabolism of *L. reuteri*. When we compared B_{12} production by cells grown in an anaerobic environment with B_{12} production by cells grown in air, combining the omission of cysteine with the addition of glycerol, it was clear that even though the trend was still observed, the increase was not as great as expected. This suggests that after the production of B_{12} is increased 20-fold, another bottleneck is encountered.

The transcriptome analysis of cells grown in the absence of cysteine compared to cells grown in complete CDM confirms that in *L. reuteri* cysteine is made from serine via cysteine synthase, using sulfur groups derived from methionine. As mentioned above, *L. reuteri* contains two copies of cysteine synthase genes (lreu_1553 and lreu_1792), which are upregulated in the absence of cysteine. We noticed that while the increased expression values for lreu_1792 are very similar for exponential- and stationary-phase data sets, the paralog, lreu_1553, seems to be preferred in the later stages of growth. All pathways from amino acids to carbomyl phosphate appeared to be downregulated, along with the operon that encodes the machinery necessary to channel carbomyl phosphate to pyrimidine biosynthesis. This is most likely related to the decrease in the growth rate that we characterized when cysteine was omitted from CDM (~15% reduction).

The presence of the B_{12} biosynthesis gene cluster among the genes that are upregulated in the absence of Cys was expected given the phenotype that we described here. The fact that we cannot determine that the full length of the cluster is upregulated can be easily explained by the large size and fragility of the transcribed mRNA species, as previously noted when Northern blot analysis was performed (14). Nonetheless, if we decrease the stringency of our established thresholds, the whole cluster, instead of just the genes in its center, has a positive M value. Based on the transcriptome data alone, de novo production of riboflavin and folate seems to be negatively affected by omission of Cys. Since excess levels of both of these vitamins are actually present in CDM and we have recently described metabolic engineering strategies to increase folate production in *L. reuteri* (15), we decided not to pursue this possibility further.

Omission of Cys has implications for sulfur metabolism that extend beyond its own biosynthesis. The ubiquity of the loci encoding thioredoxin and thioredoxin reductase (EC 1.8.1.9) in both data sets and of the glutaredoxin locus in the stationary-phase set illustrates this. These proteins are known to reduce other proteins by cysteine thiol-disulfide exchange, acting as antioxidants. Thus, there seem to be interactions that have not been clarified yet which could lead to even greater B_{12} production. A factor that is partially related to this is cobalt bioavailability. We observed upregulation of genes for proteins involved in the uptake of cobalt, which is logical since this metal is located in the core of the tetrapyrrole macrocycle of B_{12} (10). Adding more cobalt to CDM supplemented with glycerol but without cysteine did not result in a significant increase in B_{12} production (data not shown), which was not unexpected since CDM contains excess cobalt. This indicates that the cobalt supply is a limiting factor that is compensated for by increased expression of one or more cobalt transporters. The bioavailability of cobalt could be the bottleneck that limited the engineering of B_{12} production to a 20-fold increase.

Production of vitamin B_{12} relies solely on microbial production, since chemical methods are not economically viable due to the technical complexity of the synthesis process (8). *Pseudomonas denitrificans* and *Propionibacterium* subspecies account for most of the industrially produced vitamin B_{12}, and the productivity is high as 300 mg liter^{-1} (8). We have used physiological approaches to modulate the production of B_{12} in *L. reuteri* JCM1112 from nearly undetectable levels to levels 20-fold higher than the levels previously reported (15). Although the level of B_{12} produced in this study using *L. reuteri* falls short of the maximum level reported by nearly 2 orders of magnitude, *L. reuteri* possesses generally regard as safe status and is a suitable host for in situ production. Furthermore, we characterized the impact on growth behavior of all cultivation conditions tested and analyzed by using cDNA microarray experiments the transcriptional response of *L. reuteri* to omission of cysteine. This led to new insights that could be used to improve the production of vitamin B_{12} even further.

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REFERENCES

1. **Cleusix, V., C. Lacroix, S. Vollenweider, M. Duboux, and G. Le Blay.** 2007. Inhibitory activity spectrum of reuterin produced by *Lactobacillus reuteri* against intestinal bacteria. BMC Microbiol. **7:**101.
2. **Daniel, R., T. A. Bobik, and G. Gottschalk.** 1998. Biochemistry of coenzyme B_{12}-dependent glycerol and diol dehydratases and organization of the encoding genes. FEMS Microbiol. Rev. **22:**553–566.
3. **Dekel, E., and U. Alon.** 2005. Optimality and evolutionary tuning of the expression level of a protein. Nature **436:**588–592.
4. **Food and Agriculture Organization, World Health Organization.** 2004. Vitamin and mineral requirements in human nutrition, 2nd ed. World Health Organization, Geneva, Switzerland.
5. **Hodgkin, D. G., J. Pickworth, J. H. Robertson, K. N. Trueblood, R. J. Prosen, and J. G. White.** 1955. The crystal structure of the hexacarboxylic acid derived from B_{12} and the molecular structure of the vitamin. Nature **176:** 325–328.
6. **Horowitz, W. (ed.).** 2006. Official methods of analysis of AOAC International, 18th ed. AOAC International, Gaithersburg, MD.
7. **Hunik, J. H.** 2001. Non-continuous process for the production of vitamin B_{12}. European patent EP1144670.
8. **Martens, J. H., H. Barg, M. J. Warren, and D. Jahn.** 2002. Microbial production of vitamin B_{12}. Appl. Microbiol. Biotechnol. **58:**275–285.
9. **Pieterse, B., R. H. Jellema, and M. J. van der Werf.** 2006. Quenching of microbial samples for increased reliability of microarray data. J. Microbiol. Methods **64:**207–216.
10. **Rickes, E. L., N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers.** 1948. Vitamin B_{12}, a cobalt complex. Science **108:**134.
11. **Rodionov, D. A., A. G. Vitreschak, A. A. Mironov, and M. S. Gelfand.** 2003. Comparative genomics of the vitamin B_{12} metabolism and regulation in prokaryotes. J. Biol. Chem. **278:**41148–41159.
12. **Santos, F.** 2008. Vitamin B_{12} synthesis in *Lactobacillus reuteri.* Ph.D. thesis. Wageningen University, Wageningen, The Netherlands.
13. **Santos, F., J. L. Vera, P. Lamosa, G. F. de Valdez, W. M. de Vos, H. Santos, F. Sesma, and J. Hugenholtz.** 2007. Pseudovitamin B_{12} is the corrinoid produced by *Lactobacillus reuteri* CRL1098 under anaerobic conditions. FEBS Lett. **581:**4865–4870.
14. **Santos, F., J. L. Vera, R. van der Heijden, G. Valdez, W. M. de Vos, F. Sesma, and J. Hugenholtz.** 2008. The complete coenzyme B_{12} biosynthesis gene cluster of *Lactobacillus reuteri* CRL1098. Microbiology **154:**81–93.
15. **Santos, F., A. Wegkamp, W. M. de Vos, E. J. Smid, and J. Hugenholtz.** 2008. High-level folate production in fermented foods by the B_{12} producer *Lactobacillus reuteri* JCM1112. Appl. Environ. Microbiol. **74:**3291–3294.
16. **Saulnier, D. M., D. Molenaar, W. M. de Vos, G. R. Gibson, and S. Kolida.** 2007. Identification of prebiotic fructooligosaccharide metabolism in *Lactobacillus plantarum* WCFS1 through microarrays. Appl. Environ. Microbiol. **73:**1753–1765.
17. **Smyth, G. K.** 2005. Limma: linear models for microarray data, p. 397–420. *In* R. Gentleman, S. Carey, S. Dudoit, R. Irizarry, and W. Huber (ed.), Bioinformatics and computational biology solutions using R and Bioconductor. Springer, New York, NY.
18. **Smyth, G. K.** 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. **3:**Article3.
19. **Stabler, S. P.** 1999. B_{12} and nutrition, p. 343–365. *In* R. Banerjee (ed.), Chemistry and biochemistry of B_{12}. John Wiley & Sons, Inc., New York, NY.
20. **Talarico, T. L., I. A. Casas, T. C. Chung, and W. J. Dobrogosz.** 1988. Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. Antimicrob. Agents Chemother. **32:**1854–1858.
21. **Tatusov, R. L., M. Y. Galperin, D. A. Natale, and E. V. Koonin.** 2000. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res. **28:**33–36.
22. **Teusink, B., F. H. van Enckevort, C. Francke, A. Wiersma, A. Wegkamp, E. J. Smid, and R. J. Siezen.** 2005. In silico reconstruction of the metabolic pathways of *Lactobacillus plantarum*: comparing predictions of nutrient requirements with those from growth experiments. Appl. Environ. Microbiol. **71:**7253–7262.
23. **Walter, J., N. C. Heng, W. P. Hammes, D. M. Loach, G. W. Tannock, and C. Hertel.** 2003. Identification of *Lactobacillus reuteri* genes specifically induced in the mouse gastrointestinal tract. Appl. Environ. Microbiol. **69:**2044–2051.
24. **Yang, Y. H., S. Dudoit, P. Luu, D. M. Lin, V. Peng, J. Ngai, and T. P. Speed.** 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. **30:**e15.